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(54) Title: USE OF MUTANT ENTEROTOXIN WITH EXCESS B–SUBUNIT AS AN ADJUVANT

(57) Abstract

The present invention is directed towards compositions and methods which provide enhanced adjuvanticity of a genetically distinct mutant of *E. coli* heat–labile enterotoxin (LT). Specifically, the invention relates to formulations and methods for use of a mutant LT designated LT(R192G) together with an excess of LT–B–subunits. The compositions of the invention are shown to have qualitatively enhanced adjuvanticity to induce both antigen–specific antibody and T–cell responses when administered orally, and quantitatively enhanced adjuvanticity when administered intranasally, when compared to a formulation containing the mutant holotoxin without excess B–subunits.

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USE OF MUTANT ENTEROTOXIN WITH EXCESS B-SUBUNIT AS AN ADJUVANT

1. FIELD OF THE INVENTION

The present invention is directed towards compositions and methods which provide enhanced adjuvanticity of a genetically distinct mutant of *E. coli* heat-labile enterotoxin (LT). Specifically, the invention relates to formulations and methods for use of a mutant LT designated LT(R192G), modified by a single amino acid substitution that substantially reduces its inherent toxicity but leaves intact the adjuvant properties of the molecule, provided as a single mutant A-subunit with five B-subunits, *i.e.*, mutant holotoxin, together with an excess of B-subunits which is shown to have qualitatively enhanced adjuvanticity to induce both antigen-specific antibody and T-cell responses when administered orally, and quantitatively enhanced adjuvanticity when administered intranasally, when compared to a formulation containing the mutant holotoxin without excess B-subunits.

2. BACKGROUND OF THE INVENTION

The World Health Organization report of Infectious Disease deaths for 1995 indicated that there were more than 13 million deaths world-wide during that year. The majority of those deaths were caused by organisms that first make contact with and then either colonize or cross mucosal surfaces to infect the host. The overall morbidity caused by these organisms and other pathogens that interact with mucosal surfaces is impossible to calculate.

Traditional vaccine strategies that involve parenteral immunization with inactivated viruses or bacteria or subunits of relevant virulence determinants of those pathogens do not prevent those interactions. In fact,

traditional vaccine strategies do not prevent infection but instead resolve infection before disease ensues. In some cases, HIV for example, once the virus crosses the mucosal surface and enters the host cell, be that a dendritic cell, an epithelial cell, or a T-cell, the host-parasite relationship is moved decidedly in favor of the parasite (HIV). In that case, as in many others, a vaccine strategy that does not prevent the initial infection of the host is unlikely to succeed.

Recently, a great deal of attention has focused on mucosal immunization as a means of inducing secretory IgA (sIgA) antibodies directed against specific pathogens of mucosal surfaces. The rationale for this is the recognition that sIgA constitutes greater than 80% of all antibodies produced in mucosal-associated lymphoid tissues in humans and that sIgA may block attachment of bacteria and viruses, neutralize bacterial toxins, and even inactivate invading viruses inside of epithelial cells. In addition, the existence of a Common Mucosal Immune System permits immunization on or at one mucosal surface to induce secretion of antigen-specific sIgA at distant mucosal sites. It is only now being appreciated that mucosal immunization may be an effective means of inducing not only sIgA but also systemic antibody and cell-mediated immunity.

The mucosal immune response can be divided into two phases (McGhee and Kiyono, 1993, *Infect Agents Dis* 12:55-73). First, the inductive phase involves antigen presentation and the initiation events which dictate the subsequent immune response. During the initiation events, antigen-specific lymphocytes are primed and migrate from the inductive sites (*e.g.*, Peyer's patches in the enteric mucosa) through the regional lymph nodes, into the circulation and back to mucosal effector sites (*e.g.*, lamina propria). Once these effector cells have seeded their effector sites, the second

phase, or effector phase, of the mucosal immune response can occur. A significant difference between mucosal immunization and parenteral immunization is that both mucosal and systemic immunity can be induced by mucosal immunization while parenteral immunization generally results only in systemic responses.

Most studies conducted to date have dealt with the secretory antibody component of the mucosal response and the complex regulatory issues involved with induction of sIgA following mucosal immunization and not with the systemic antibody response or cellular immunity induced by mucosal immunization. In that regard, it is important to understand the type of helper T lymphocyte response induced by mucosal immunization since the type of helper T lymphocyte stimulated by an antigen is one of the most important factors for defining which type of immune response will follow. Mosmann and colleagues (Cherwinski et al., 1987, *Journal of Experimental Medicine* 166:1229-1244; Mosmann and Coffman, 1989, *Annual Reviews of Immunology* 7:145-173) discovered that there are at least two different types of helper T lymphocytes (Th) which can be identified based on cytokine secretion. Th1 lymphocytes secrete substantial amounts of IL-2 and INF-gamma and execute cell-mediated immune responses (*e.g.*, delayed type hypersensitivity and macrophage activation), whereas Th2 lymphocytes secrete IL-4, IL-5, IL-6 and IL-10 and assist in antibody production for humoral immunity. Theoretically then, antigenic stimulation of one T helper cell subset and not the other would result in production of a particular set of cytokines which would define the resulting immune response.

The presence of IL-2 and INF-gamma coupled with an antigenic stimulus presented by macrophages in the context of Class II MHC molecules can initiate Th1 type responses. The ability of Th1 cells to secrete IL-2 and INF-gamma further

amplifies the response by activating Th1 cells in an autocrine fashion and macrophages in a paracrine fashion. These activated leukocytes can release additional cytokines (*e.g.*, IL-6) which may induce the proliferation and differentiation of antigen specific B lymphocytes to secrete antibody (the effector phase). In this scenario, the predominant isotype secreted by murine B lymphocytes is often IgG2a. In a second scenario (Urban et al., 1992, *Immunol Rev* 127:205-220), antigens such as allergens or parasites can effectively stimulate a Th2 lymphocyte response (the inductive phase). Presentation of such antigens to Th2 cells can result in the production of the lymphokines IL-4 and IL-5 which can induce antigen specific B lymphocytes to secrete IgE and IgG1 or induce eosinophillia, respectively (the effector phase). Furthermore, stimulated Th2 cells can secrete IL-10 which has the ability to specifically inhibit secretion of IL-2 and INF-gamma by Th1 lymphocytes and also to inhibit macrophage function.

While these representations are simplistic, it is obvious that the type of T helper cell stimulated affects the resultant cellular immune response as well as the predominant immunoglobulin isotype secreted. Specifically, IL-4 stimulates switching to the IgE and IgG1 isotypes whereas INF-gamma stimulates IgG2a secretion. Numerous studies, predominantly conducted in vitro, have suggested that IL-5, IL-6 and TGF-beta (Th3) can cause isotype switching to IgA.

2.1. MUCOSAL ADJUVANTS

Mucosally administered antigens are frequently not immunogenic. A number of strategies have been developed to facilitate mucosal immunization, including the use of attenuated mutants of bacteria (*e.g.*, *Salmonella spp.*) as carriers of heterologous antigens, encapsulation of antigens into microspheres, gelatin capsules, different formulations

of liposomes, adsorption onto nanoparticles, use of lipophilic immune stimulating complexes, and addition of bacterial products with known adjuvant properties. The two bacterial products with the greatest potential to function as mucosal adjuvants are cholera toxin (CT), produced by various strains of *Vibrio cholerae*, and the heat-labile enterotoxin (LT) produced by some enterotoxigenic strains of *Escherichia coli* (Clements et al., 1988, *Vaccine* 6:269-277; Elson, 1989, *Immunology Today* 146:29-33; Lycke et al., 1992, *European Journal of Immunology* 22:2277-2281; Xu-Amano et al., 1993, *Journal of Experimental Medicine* 178:1309-1320; Yamamoto et al., 1996, *Annals of the New York Academy of Sciences* 778:64-71).

Although LT and CT have many features in common, these are clearly distinct molecules with biochemical and immunologic differences which make them unique (see below). Both LT and CT are synthesized as multisubunit toxins with A and B components. On thiol reduction, the A component dissociates into two smaller polypeptide chains. One of these, the A1 piece, catalyzes the ADP-ribosylation of the stimulatory GTP-binding protein (GSa) in the adenylate cyclase enzyme complex on the basolateral surface of the epithelial cell resulting in increasing intracellular levels of cAMP. The resulting increase in cAMP causes secretion of water and electrolytes into the small intestine through interaction with two cAMP-sensitive ion transport mechanisms involving 1) NaCl cotransport across the brush border of villous epithelial cells, and 2) electrogenic Na dependent Cl secretion by crypt cells (Field, 1980, *Secretory Diarrhea* pp21-30). The B-subunit binds to the host cell membrane receptor (ganglioside GM1) and facilitates the translocation of the A-subunit through the cell membrane.

Recent studies have examined the potential of CT and LT as a mucosal adjuvant against a variety of bacterial

and viral pathogens using whole killed organisms or purified subunits of relevant virulence determinants from these organisms. Representative examples include tetanus toxoid (Xu-Amano et al., 1993, *Journal of Experimental Medicine* 178:1309-1320; Yamamoto et al., 1996, *Annals of the New York Academy of Sciences* 778:64-71; Xu-Amano et al., 1994, *Vaccine* 12:903-911), inactivated influenza virus (Hashigucci et al., 1996, *Vaccine* 14:113-119; Katz et al., 1996, *Options for the control of influenza. III.*, pp292-297; Katz et al., 1997, *Journal of Infectious Diseases* 175:352-363), recombinant urease from *Helicobacter spp.* (Lee et al., 1995, *Journal of Infectious Diseases* 172:161-171; Weltzin et al., 1997, *Vaccine* 4:370-376), pneumococcal surface protein A from *Streptococcus pneumoniae* (Wu et al., 1997, *Journal of Infectious Diseases* 175:839-846), Norwalk virus capsid protein, synthetic peptides from measles virus (Hathaway et al., 1995, *Vaccine* 13:1495-1500), and the HIV-1 C4/V3 peptide T1SP10 MN(A) (Staats et al., 1996, *Journal of Immunology* 157:462-472). There are many other examples and it is clear that both LT and CT have significant potential for use as adjuvants for mucosally administered antigens (*see* Dickinson and Clements, 1996, *Mucosal Vaccines* pp73-87 for a recent review). This raises the possibility of an effective immunization program against a variety of pathogens involving the mucosal administration of killed or attenuated agents or relevant virulence determinants of specific agents in conjunction with LT or CT. However, the fact that these "toxins" can stimulate a net lumenal secretory response may prevent their use. For instance, as little as 5 μg of purified CT was sufficient to induce significant diarrhea in volunteers while 25 μg was shown to elicit a full 20-liter cholera purge (Levine et al., 1983, *Microbiological Reviews* 47:510-550). In recently conducted volunteer studies with LT administered alone or in conjunction with the *V. cholerae*

Whole Cell/B-Subunit Vaccine, LT was shown to induce fluid secretion at doses as low as 2.5 μg when administered in conjunction with the vaccine, while 25 μg of LT elicited up to 6-liters of fluid. While the adjuvant effective dose in humans for either of these toxins has not been established, experiments in animals suggest that it may be a comparable to the toxic dose. Taken together, these studies indicate that while LT and CT may be attractive as mucosal adjuvants, studies in animals do not reflect the full toxic potential of these molecules in humans, and that toxicity will seriously limit their practical use for humans.

A number of attempts have been made to alter the toxicity of LT and CT, most of which have focused on eliminating enzymatic activity of the A-subunit associated with enterotoxicity. The majority of these efforts have involved the use of site-directed mutagenesis to change amino acids associated with the crevice where NAD binding and catalysis is thought to occur. Recently, a model for NAD binding and catalysis was proposed (Domenighini et al., 1994, *Molecular Microbiology* 14:41-50; Pizza et al., 1994, *Molecular Microbiology* 14:51-60) based on computer analysis of the crystallographic structure of LT (Sixma et al., 1991, *Nature* (London) 351:371-377; Sixma et al., 1993, *Journal of Molecular Biology* 230:890-918). Replacement of any amino acid in CT or LT involved in NAD-binding and catalysis by site-directed mutagenesis has been shown to alter ADP-ribosyltransferase activity with a corresponding loss of toxicity in a variety of biological assay systems (Lycke et al., 1992, *European Journal of Immunology* 22:2277-2281; Burnette et al., 1991, *Infection and Immunity* 59:4266-4270; Harford et al., 1989, *European Journal of Biochemistry* 183:311-316; Häse et al., 1994, *Infection and Immunity* 62:3051-3057; Lobet et al., 1991, *Infection and Immunity* 59:2870-2879; Merritt et al., 1995, *Nature Structural Biology*

2:269-272; Moss et al., 1993, *Journal of Biological Chemistry* 268:6383-6387; Tsuji et al., 1991, *FEBS Letters* 291:319-321; Tsuji et al., 1990, *Journal of Biological Chemistry* 265:22520-22525). In addition, it has been shown that exchanging K for E112 in LT not only removes ADP-ribosylating enzymatic activity, but cAMP activation and adjuvant activity as well (Lycke et al., 1992, *European Journal of Immunology* 22:2277-2281). A logical conclusion from the Lycke et al. studies was that ADP-ribosylation and induction of cAMP are essential for the adjuvant activity of these molecules. As a result, a causal linkage was established between adjuvanticity and enterotoxicity. That is, the accumulation of cAMP responsible for net ion and fluid secretion into the gut lumen was thought to be a requisite to adjuvanticity. Recent studies by a number of laboratories have challenged that linkage.

Dickinson and Clements (Dickinson and Clements, 1995, *Infection and Immunity* 63:1617-1623) (Clements et al.) explored an alternate approach to dissociation of enterotoxicity from adjuvanticity. LT requires proteolysis of a trypsin sensitive bond to become fully active. In this enterotoxin, that trypsin sensitive peptide is subtended by a disulfide interchange that joins the A1 and A2 pieces of the A-subunit. In theory, if the A1 and A2 pieces cannot separate, A1 will not be able to find its target (adenylate cyclase) on the basolateral surface or assume the conformation necessary to bind or hydrolyze NAD.

The mutant of Clements et al. has been described more fully in PCT Publication WO96/06627, incorporated herein by reference. The mutant LT holotoxin, designated LT(R192G), was constructed using site-directed mutagenesis to create a single amino acid substitution within the disulfide subtended region of the A-subunit separating A1 from A2. This single amino acid change altered the proteolytically sensitive site

within this region, rendering the mutant insensitive to trypsin activation. The physical characteristics of this mutant were examined by SDS-PAGE, its biological activity was examined on mouse Y-1 adrenal tumor cells and Caco-2 cells, its enzymatic properties determined in an in vitro NAD:agmatine ADP-ribosyltransferase assay, and its immunogenicity and immunomodulating capabilities determined by testing for the retention of immunogenicity and adjuvanticity.

PROPERTIES OF LT(R192G)

- 100 - 1,000 fold less active than cholera toxin or native LT in the mouse Y-1 adrenal cell assay
- Not sensitive to proteolytic activation
- Does not possess in vitro NAD:agmatine ADP-ribosyltransferase activity
- Does not increase production of cAMP in cultured Caco-2 cells
- Reduced enterotoxicity in the patent mouse intestinal challenge model when compared to native LT
- Promotes the development of both humoral (antibody) and cell-mediated immune responses against co-administered antigens of a pathogenic microorganism in both the systemic and mucosal compartments
- Functions as an effective adjuvant when administered mucosally (i.e., orally, intranasally) or parenterally (i.e., subcutaneously)
- Lacks enterotoxicity in humans at adjuvant-effective doses

WO 96/06627 describes plasmid pBD95 which can be used to obtain the mutant LT(R192G). Although not described in WO 96/06627, it has recently been discovered that when plasmid pBD95 is used to produce the mutant holotoxin,

LT(R192G), by expressing pBD95 in *E. coli*, varying amounts of free B-subunit can also be recovered as well as the holotoxin. This phenomenon is well known to those of skill in the art since some excess B-subunit is always present following purification of LT or CT by galactose affinity chromatography. Pizza et al. (Pizza et al., 1994, Molecular Microbiology 14:51-61) report mutant and wild-type AB5/AB5+B5 ratios that vary from 40% to 98% depending upon the type of mutation. Such excess B-subunit can be separated from holotoxin by gel filtration chromatography due to the difference in molecular weight between the holotoxin and the free B-subunit pentamer (84 kd vs. 56 kd).

The mutant LT composition produced using pBD95 in *E. coli* induces an immune response which includes both humoral and T-cell components.

LT(R192G) has been shown to possess the capability of enhancing an immune response (*e.g.*, IgG, IgA) to antigens unrelated to LT or LT(R192G). Recent experimental evidence shows that LT(R192G) has utility as an adjuvant for mucosally or parenterally administered antigens; such administration results in the production of serum IgG and/or mucosal sIgA as well as cell-mediated immune responses against the antigen with which LT(R192G) is delivered and, more importantly, to protect against subsequent challenge with infectious organisms. LT(R192G) has been shown to be an effective mucosal adjuvant and has recently been evaluated in humans in several Phase I safety studies.

More recently, Tsuji et al. (Tsuji et al., 1997, *Immunology* 90:176-182) demonstrated that a protease-site deletion mutant LT(Δ192-194) also lacks in vitro ADP-ribosylagmatine activity, has a ten-fold reduction in enterotoxicity in rabbit ligated ileal loops, and a 50% reduction and delayed onset of cAMP induction in cultured myeloma cells. LT(Δ192-194) was shown to have increased

adjuvant activity for induction of serum IgG and mucosal IgA against measles virus when compared to native LT, LT-B, or LT(E112K). LT(Δ192-194) was effective when administered intranasally, subcutaneously, intraperitoneally, or orally although mucosal IgA responses were only demonstrated following mucosal administration. These investigators also demonstrated increased adjuvant activity for mucosally administered LT(Δ192-194) in conjunction with KLH, BCG, and Ova. These findings are consistent with the findings with LT(R192G).

2.2. THE ROLE OF FREE B-SUBUNIT

There have been occasional reports that the isolated B-subunit of LT exhibits adjuvanticity when administered intranasally, but not orally. In most studies, however, the isolated B-subunits of LT does not exhbiit adjuvanticity. One view is that isolated recombinant B-subunit does not have adjuvant activity. Where activity has been observed for isolated B-subunit, it has typically been with B-subunit prepared from LT holotoxin by dissociation chromatography by gel filtration in the presence of a dissociating agent (*i.e.*, guanidine HCl or formic acid). The isolated subunits are then pooled and the dissociating agent removed. B-subunit prepared by this technique is invariably contaminated with trace amounts of A-subunit such that upon renaturation a small amount of holotoxin is reconstituted. The reports of Yamamoto et al. (Yamamoto et al., 1997, *Journal of Experimental Medicine* 185:1203-1210; Yamamoto et al., 1997, *Proceedings of the National Academy of Sciences* 94:5267-5272) with recombinant CT-B, free of contaminating A-subunit as well as studies with recombinant LT-B (Clements et al., 1988, *Vaccine* 6:269-277) support that conclusion. It is not, however, a universally accepted conclusion.

There have been a number of studies conducted in which the B-subunit of either CT or LT has been shown to have adjuvanticity when admixed with a trace amount of holotoxin (see, for example, Hashigucci et al., 1996, *Vaccine* 14:113-119; Hathaway et al., 1995, *Vaccine* 13:1495-1500).

In the Hashigucci et al. study, (Hathaway et al., 1995, *Vaccine* 13:1495-1500), LT-B with 0.5% LT holotoxin was shown to function as an immunologic adjuvant for influenza virus vaccine when administered intranasally. This represents a B-subunit to LT holotoxin ratio of 200:1 and no effect on toxicity was determined. Moreover, there was no indication that free B-subunit qualitatively changes the outcome when admixed with native LT.

One report has shown increased adjuvanticity against influenza virus by nasal administration of a composition of influenza hemagglutinin and a mutant holotoxin, in which the Arg at position 7 was changed to Lys, with additional LT B-subunit. (Komase et al., 1998, *Vaccine* 16(2/3):248-254).

It is an object of the invention to provide novel and improved compositions and methods for use of LT(R192G) with excess B-subunit which have advantageously surprising benefits providing enhanced adjuvanticity of an antigen administered either orally or nasally. In addition, the methods and compositions provide a qualitatively enhanced immunological outcome when administered orally.

Citation or identification of any reference in Section 2 or any section of this application shall not be construed as an admission that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention is based on the surprising discovery that an amount of free B-subunit of the heat-labile

enterotoxin of *E. coli* (LT-B) in combination with a protease-site mutant of LT, designated LT(R192G), was found to qualitatively and quantitatively enhance the immunological outcome when LT(R192G) was used in combination with excess B-subunit as an oral adjuvant, and to quantitatively enhance the immunological outcome when the combination was used as a nasal adjuvant.

The present invention also provides a method for further enhancing the immune response to a co-administered antigen when LT(R192G) is used as an oral adjuvant by including an excess amount of free B-subunit of LT.

The invention also provides a composition useful in these methods. The composition comprises an effective amount of LT(R192G) in combination with free B-subunit of LT and an effective amount of antigen.

The present invention supersedes the prior art in that LT(R192G) in the presence of free B-subunit has enhanced adjuvanticity for both antigen-specific antibody and T-cell responses when administered orally and enhanced adjuvanticity when administered intranasally in comparison to LT(R192G) holotoxin without excess free B-subunit. The utility of this surprising discovery is that an adjuvant effective amount of LT(R192G) may be utilized in an effective immunization program against a variety of pathogens involving the administration of an effective amount of LT(R192G) adjuvant plus excess B-subunit in admixture with killed or attenuated pathogens or relevant virulence determinants of specific pathogens.

The present invention further supersedes the prior art in that the present invention may be used to specifically increase levels of antigen-specific Th1- and Th2-type cytokines and serum antibody responses when LT(R192G) in combination with excess free B-subunit of LT is used an oral adjuvant. This finding is totally unexpected, given current

understanding of immune cross-regulation between the Th1 and Th2 arms of the immune response. One skilled in the art would generally expect an increasing Th1 response to down-regulate the Th2 response. The fact that this was not the case when LT(R192G) was used as an adjuvant was unexpected.

4. DEFINITIONS

As used herein, the term "holotoxin" refers to a complex of five B-subunits and one A-subunit of heat-labile enterotoxin.

As used herein, the term "free B-subunit" refers to the B-subunit of heat-labile enterotoxin substantially free from the A-subunit of heat-labile enterotoxin.

As used herein, the term "excess B-subunit" refers to an amount of B-subunit which results in greater than a 5:1 ratio of B-subunits to A-subunit, 5:1 being the ratio of B:A subunits present in native holotoxin, i.e. natural heat-labile enterotoxin.

As used herein, the term "qualitatively enhanced" refers to an immune response which differs from the type of response elicited by adjuvant and immunogen without excess B-subunit. For example, when administered orally, immunogen with LT(R192G) with excess B-subunit elicits an enhanced T-cell response as compared to immunogen and LT(R192G) without excess B-subunit, which elicits a mostly humoral response.

As used herein, the term "quantitatively enhanced" refers to an immune response which is greater than normal, but does not differ in the type of immune response elicited. In one embodiment, adjuvanticity of LT(R192G) is enhanced four fold, such that only one fourth the amount of LT(R192G) with excess B-subunit is required, as compared to LT(R192G) without excess B-subunit, to elicit a comparable immune response.

5. BRIEF DESCRIPTION OF THE FIGURES

The present invention may be understood more fully by reference to the following detailed description of the invention, examples of specific embodiments of the invention and the appended figures in which:

Figure 1 is a schematic diagram of plasmid pCS95, which encodes both subunits LT A and B under the control of the *lac* promoter. Figure 1A illustrates the construction of plasmid pCS95 which contains the nucleotide sequence encoding mutant LT(R192G). Plasmid pCS95 was constructed by replacing the BamHI-XbaI of pBD95 with the BamHI-XbaI fragment of pDF82. Figure 1B shows the single amino acid change in LT(R192G). Plasmid pCS95 provides LT(R192G) which contains the single base substitution at amino acid residue 192 of subunit A, coding for Gly rather than Arg, which preserves the reading frame but eliminates the proteolytic site.

Figure 2 is a graphic illustration of the effect of various ratios of free B-subunit to LT(R192G) in the patent mouse intestinal assay. For these studies, LT(R192G) with no excess B-subunit was admixed with different ratios of B-subunit and examined for toxicity in the patent mouse assay. Groups of mice were orally inoculated with native LT at 5, 25, 50 or 100 μg, or with 25 μg of LT(R192G) admixed with a different amount of free B-subunit. Following a three hour interval, the gut:carcass ratio of each animal was determined. The gut-carcass ratio is defined as the intestinal weight divided by the remaining carcass weight. There were three animals per group and the means for each data point are shown.

Figure 3 is an additional graphic illustration of the effect of excess B-subunit in the patent mouse intestinal assay. For these studies, groups of mice were orally inoculated with native LT at 5, 25, or 125 μg, or with 25 μg of LT(R192G). Other groups received either 25 μg of native

LT or 25 μg of LT(R192G) admixed with a 3:1 or 10:1 excess of free B-subunit. Following a three hour interval, the gut:carcass ratio of each animal was determined. There were three animals per group and the means for each data point are shown.

Figure 4 is a graphic illustration of the effect of excess B-subunit on the ability of LT(R192G) to function as an immunologic adjuvant for induction of serum IgG when administered intranasally. Mice were immunized intranasally with Ovalbumin (Ova) alone or in conjunction with 5 μg of LT(R192G) or 1.25 μg of LT(R192G) plus 3.75 μg of excess free B-subunit, designated 1AB5:3B5. Serum anti-Ova IgG was determined by ELISA. There were seven animals per group and the means for each data point are shown.

Figure 5 is a graphical illustration of the effect of excess B-subunit on the ability of LT(R192G) to function as an immunologic adjuvant for production of antigen-specific Th1-type cytokines, specifically, IFN-gamma, by mononuclear cells from the spleens of animals immunized intranasally. Mice were immunized intranasally with Ovalbumin (Ova) alone or in conjunction with 5 μg of LT(R192G) or 1.25 μg of LT(R192G) plus 3.75 μg of excess free B-subunit, designated 1AB5:3B5. Cytokines were determined by ELISA following a T-cell restimulation assay.

Figure 6 is a graphic illustration of the effect of excesss B-subunit on the ability of LT(R192G) to function as an immunologic adjuvant for production of antigen-specific Th2-type cytokines, specifically, IL-10, by mononuclear cells from the spleens of animals immunized intranasally. Mice were immunized intranasally with Ovalbumin (Ova) alone or in conjunction with 5 μg of LT(R192G)-AB5 or 1.25 μg of LT(R192G) plus 3.75 μg of excess free B-subunit, designated 1AB5:3B5. Cytokines were determined by ELISA following a T-cell restimulation assay.

Figure 7 is a graphical demonstration that excess B-subunit enhances the ability of LT(R192G) to function as an immunologic adjuvant for induction of serum IgG when administered orally. Mice were immunized orally with a purified bacterial protein, Colonizing Factor I (CFAI) from enterotoxigenic *E. coli*, in conjunction with 6.25 μg of LT(R192G) or 6.25 μg of LT(R192G) plus 18.75 μg of free B-subunit, designated 1AB5:3B5. Serum anti-CFAI IgG was determined by ELISA. There were seven animals per group and the means for each data point are shown.

Figure 8 is an additional graphic illustration that excess B-subunit enhances the ability of LT(R192G) to function as an immunologic adjuvant for induction of serum IgG when administered orally. Mice were immunized orally with Ovalbumin (Ova) alone or in conjunction with 25 μg of LT(R192G) or 6.25 μg of LT(R192G) plus 18.75 μg of free B-subunit, designated 1AB5:3B5. Serum anti-Ova IgG was determined by ELISA. There were ten animals per group and the means for each data point are shown.

Figure 9 is a graphic demonstration that excess B-subunit enhances the ability of LT(R192G) to function as an immunologic adjuvant for production of antigen-specific Th1-type cytokines, specifically, IFN-gamma, by mononuclear cells from the spleens of animals immunized orally. Mice were immunized orally with Ovalbumin (Ova) alone or in conjunction with 25 μg of LT(R192G) or 6.25 μg of LT(R192G) plus 18.75 μg of free B-subunit, designated 1AB5:3B5. Cytokines were determined by ELISA following a T-cell restimulation assay.

Figure 10 is a graphic demonstration that excess B-subunit enhances the ability of LT(R192G) to function as an immunologic adjuvant for production of antigen-specific Th2-type cytokines, specifically, IL-10, by mononuclear cells from the spleens of animals immunized orally. Mice were

immunized orally with Ovalbumin (Ova) alone or in conjunction with 25 μg of LT(R192G) or 6.25 μg of LT(R192G) plus 18.75 μg of free B-subunit, designated 1AB5:3B5. Cytokines were determined by ELISA following a T-cell restimulation assay.

6. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel compositions of LT(R192G) combined with free B-subunit and compositions thereof, and methods of using LT(R192G) and free B-subunit as an adjuvant having advantages for use to induce an immune response to a co-administered antigen.

6.1. PRODUCTION OF LT(R192G)

LT(R192G) can be produced by a number of means apparent to those of skill in the art. For example, LT(R192G) can be isolated from *E. coli* expressing pBD95, a plasmid fully described in PCT Publication WO96/06627. Subsequent to the effective priority date of WO96/06627, others have had success in isolating LT(R192G) from *E. coli* expressing other plasmid constructs. (Grant et al., (1994), *Infection and Immunity* 62(10):4270-4278). Plasmid pCS95, fully described in Example 6.1 can also be utilized to produce isolated or substantially pure LT(R192G) in *E. coli*.

LT(R192G) can be isolated by agarose affinity chromatography from bacteria expressing an LT(R192G) encoding plasmid. Alternate methods of purification will be apparent to those skilled in the art.

LT(R192G) produced by any means can be further purified by gel filtration chromatography, which allows for the separation of holotoxin from any free A or B subunits.

6.2. PRODUCTION OF LT-B

The B-subunit of LT can be produced by a number of means apparent to those of skill in the art. For example, B-

subunit can be isolated from *E. coli* expressing pJC217, a plasmid fully described in U.S. Patent No. 5,308,835. LT-B has also been isolated from bacteria expressing other plasmid constructs. For examples, see European Patent Application Serial No. 0060129; Yamamoto et al., 1981, *J. Bacteriol.* 148:983; or Sanchez et al., 1982, *FEMS Microbiol. Lett.* 14:1.

LT-B can be obtained from holotoxin obtained from *E. coli* or recombinantly expressed or from recombinantly expressed B subunit only.

LT-B can be purified by agarose affinity chromatography from bacteria expressing any plasmid encoding the B-subunit of LT. Alternate methods of purification will be apparent to those skilled in the art.

6.3. COMPOSITIONS OF LT(R192G) AND FREE B-SUBUNIT

The present invention encompasses compositions and methods for use of the compositions to promote the production of serum and/or mucosal antibodies as well as cell-mediated immune responses against an antigen that is simultaneously administered with a genetically modified bacterial toxin, *i.e.*, LT(R192G), in combination with free B-subunit.

Administration of excess B-subunit results in enhanced production of serum IgG and/or mucosal sIgA as well as cell-mediated immune responses against the antigens with which LT(R192G) is delivered.

Formulation of the compositions of the present invention is carried out through the mixing of a substantially pure preparation of LT(R192G) and LT-B subunit in amounts which yield the desired ratio of B-subunit to LT(R192G). In one embodiment, the LT(R192G) in combination with free B-subunit is at a weight ratio of 1:1 to 100:1 of B-subunit to LT(R192G). In a particular embodiment, the LT(R192G) in combination with free B-subunit is at a weight ratio of 2:1 to 10:1 of B-subunit to LT(R192G). In another

embodiment, the LT(R192G) in combination with free B-subunit is at a weight ratio of about 3:1 of B-subunit to LT(R192G).

Since LT(R192G) has been shown to function as an effective adjuvant when administered on different mucosal surfaces, the effect of free B-subunit on both intranasal and oral adjuvanticity was examined. The outcome of those studies revealed that LT(R192G) in the presence of free B-subunit had quantitatively enhanced adjuvanticity when administered intranasally and, surprisingly, both quantitatively and qualitatively enhanced adjuvanticity when administered orally.

6.4. MODE OF ADMINISTRATION OF LT(R192G) FREE B-SUBUNIT, AND UNRELATED ANTIGENS

In accordance with the present invention, LT(R192G) in combination with B-subunit free of holotoxin at any B-subunit to LT(R192G) ratio of 1:1 or greater is administered in conjunction with any biologically relevant antigen and/or vaccine, such that an increased immune response to said antigen and/or vaccine is achieved.

In a preferred embodiment, the LT(R192G) plus free B-subunit and antigen are administered simultaneously in a pharmaceutical composition comprising an effective amount of LT(R192G) plus free B-subunit and an effective amount of antigen.

In an alternative embodiment, the antigen, the LT(R192G), and the free B-subunit free of holotoxin are administered separately within a short time of each other.

In another embodiment, the antigen is administered separately within a short time of the simultaneous administration of the LT(R192G) and the B-subunit free of holotoxin.

In all embodiments, the LT(R192G) administered in combination with free B-subunit is at a ratio of between 1:1

and 100:1 of B-subunit to LT(R192G). In a particular embodiment, the LT(R192G) administered in combination with free B-subunit is at a weight ratio of 2:1 to 10:1 of B-subunit to LT(R192G). In another embodiment, the LT(R192G) administered in combination with free B-subunit is at a weight ratio of about 3:1 of B-subunit to LT(R192G).

The mode of administration is mucosal (*i.e.*, intranasal, oral, rectal) or parenteral (*i.e.*, subcutaneous, intramuscular, intradermal, intravenous, intraperitoneal). The respective amounts of LT(R192G) plus free B-subunit and antigen will vary depending upon the identity of the route of administration, antigen employed and the species of animal to be immunized. In one embodiment, the initial administration of LT(R192G) plus free B-subunit and antigen is followed by a boost of the relevant antigen. In another embodiment no boost is given. The timing of boosting may vary, depending on the route, antigen and the species being treated. The modifications in route, dosage range and timing of boosting for any given species and antigen are readily determinable by routine experimentation. The boost may be of antigen alone or in combination with LT(R192G) plus free B-subunit.

6.5. ANTIGENS USEFUL IN THE INVENTION

The methods and compositions of the present invention are intended for use both in immature and mature vertebrates, in particular birds, mammals, and humans. Useful antigens, as examples and not by way of limitation, include antigens from pathogenic strains of bacteria (*Streptococcus pyogenes, Streptococcus pneumoniae, Neisseria gonorrhoea, Neisseria meningitidis, Corynebacterium diphtheriae, Clostridium botulinum, Clostridium perfringens, Clostridium tetani, Haemophilus influenzae, Klebsiella pneumoniae, Klebsiella ozaenae, Klebsiella rhinoscleromotis, Staphylococcus aureus, Vibrio cholerae, Escherichia coli,*

Pseudomonas aeruginosa, Campylobacter jejuni, Aeromonas hydrophila, Bacillus cereus, Edwardsiella tarda, Yersinia enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Salmonella typhimurium, Treponema pallidum, Treponema pertenue, Treponema carateneum, Borrelia vincentii, Borrelia burgdorferi, Leptospira icterohemorrhagiae, Mycobacterium tuberculosis, Toxoplasma gondii, Pneumocystis carinii, Francisella tularensis, Brucella abortus, Brucella suis, Brucella melitensis, Mycoplasma spp., Rickettsia prowazeki, Rickettsia tsutsugumushi, Chlamydia spp., Helicobacter pylori); pathogenic fungi (*Coccidioides immitis, Aspergillus fumigatus, Candida albicans, Blastomyces dermatitidis, Cryptococcus neoformans, Histoplasma capsulatum*); protozoa (*Entomoeba histolytica, Trichomonas tenas, Trichomonas hominis, Trichomonas vaginalis, Trypanosoma gambiense, Trypanosoma rhodesiense, Trypanosoma cruzi, Leishmania donovani, Leishmania tropica, Leishmania braziliensis, Pneumocystis pneumonia, Plasmodium vivax, Plasmodium falciparum, Plasmodium malaria*); or Helminiths (*Enterobius vermicularis, Trichuris trichiura, Ascaris lumbricoides, Trichinella spiralis, Strongyloides stercoralis, Schistosoma japonicum, Schistosoma mansoni, Schistosoma haematobium,* and hookworms) either presented to the immune system in whole cell form or in part isolated from media cultures designed to grow said organisms which are well know in the art, or protective antigens from said organisms obtained by genetic engineering techniques or by chemical synthesis.

Other relevant antigens include pathogenic viruses (as examples and not by limitation: Poxviridae, Herpesviridae, Herpes Simplex virus 1, Herpes Simplex virus 2, Adenoviridae, Papovaviridae, Enteroviridae, Picornaviridae, Parvoviridae, Reoviridae, Retroviridae,

influenza viruses, parainfluenza viruses, mumps, measles, respiratory syncytial virus, rubella, Arboviridae, Rhabdoviridae, Arenaviridae, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis E virus, Non-A/Non-B Hepatitis virus, Rhinoviridae, Coronaviridae, Rotoviridae, and Human Immunodeficiency Virus) either presented to the immune system in whole or in part isolated from media cultures designed to grow such viruses which are well known in the art or protective antigens therefrom obtained by genetic engineering techniques or by chemical synthesis.

Further examples of relevant antigens include, but are not limited to, vaccines. Examples of such vaccines include, but are not limited to, influenza vaccine, pertussis vaccine, diphtheria and tetanus toxoid combined with pertussis vaccine, hepatitis A vaccine, hepatitis B vaccine, hepatitis C vaccine, hepatitis E vaccine, Japanese encephalitis vaccine, herpes vaccine, measles vaccine, rubella vaccine, mumps vaccine, mixed vaccine of measles, mumps and rubella, papillomavirus vaccine, parvovirus vaccine, respiratory syncytial virus vaccine, Lyme disease vaccine, polio vaccine, varicella vaccine, gonorrhea vaccine, schistosomiasis vaccine, rotavirus vaccine, mycoplasma vaccine pneumococcal vaccine, meningococcal vaccine, campylobacter vaccine, helicobacter vaccine, cholera vaccine, enterotoxigenic *E. coli* vaccine, enterohemmorgagic *E. coli* vaccine, shigella vaccine, salmonella vaccine and others. These can be produced by known common processes. In general, such vaccines comprise either the entire organism or virus grown and isolated by techniques well known to the skilled artisan or comprise relevant antigens of these organisms or viruses which are produced by genetic engineering techniques or chemical synthesis. Their production is illustrated by, but not limited to, as follows:

Influenza vaccine: a vaccine comprising the whole or part of hemagglutinin, neuraminidase, nucleoprotein and matrix protein which are obtainable by purifying a virus, which is grown in embryonated eggs, with ether and detergent, or by genetic engineering techniques or chemical synthesis.

Pertussis vaccine: a vaccine comprising the whole or a part of pertussis toxin, hemagglutinin and K-agglutinin which are obtained from avirulent toxin with formalin which is extracted by salting-out or ultracentrifugation from the culture broth or bacterial cells of Bordetella pertussis, or by genetic engineering techniques or chemical synthesis.

Diphtheria and tetanus toxoid combined with pertussis vaccine: a vaccine mixed with pertussis vaccine, diphtheria and tetanus toxoid.

Japanese encephalitis vaccine: a vaccine comprising the whole or part of an antigenic protein which is obtained by culturing a virus intracerebrally in mice and purifying the virus particles by centrifugation or ethyl alcohol and inactivating the same, or by genetic engineering techniques or chemical synthesis.

Hepatitis B vaccine: a vaccine comprising the whole or part of an antigen protein which is obtained by isolating and purifying the HBs antigen by salting-out or ultracentrifugation, obtained from hepatitis carrying blood, or by genetic engineering techniques or by chemical synthesis.

Measles vaccine: a vaccine comprising the whole or part of a virus grown in a cultured chick embryo cells or embryonated egg, or a protective antigen obtained by genetic engineering or chemical synthesis.

Rubella vaccine: a vaccine comprising the whole or part of a virus grown in cultured chick embryo cells or embryonated egg, or a protective antigen obtained by genetic engineering techniques or chemical synthesis.

Mumps vaccine: a vaccine comprising the whole or part of a virus grown in cultured rabbit cells or embryonated egg, or a protective antigen obtained by genetic engineering techniques or chemical synthesis.

Mixed vaccine of measles, rubella and mumps: a vaccine produced by mixing measles, rubella and mumps vaccines.

Rotavirus vaccine: a vaccine comprising the whole or part of a virus grown in cultured MA 104 cells or isolated from the patient's feces, or a protective antigen obtained by genetic engineering techniques or chemical synthesis.

Mycoplasma vaccine: a vaccine comprising the whole or part of mycoplasma cells grown in a liquid culture medium for mycoplasma or a protective antigen obtained by genetic engineering techniques or chemical synthesis.

Those conditions for which effective prevention may be achieved by the present method will be obvious to the skilled artisan.

The vaccine preparation compositions of the present invention can be prepared by mixing the above illustrated antigens and/or vaccines with LT(R192G) and excess free B-subunit at a desired ratio. Pyrogens or allergens should naturally be removed as completely as possible. The antigen preparation of the present invention can be used by preparing the antigen per se and the LT(R192G) together with excess free B-subunit separately or together.

Further, the present invention encompasses a kit comprising an effective amount of antigen and an adjuvant effective amount of LT(R192G) plus excess free B-subunit. In use, the components of the kit can either first be mixed together and then administered or the components can be administered separately within a short time of each other.

The vaccine preparation compositions of the present invention can be combined with either a liquid or solid

pharmaceutical carrier, and the compositions can be in the form of tablets, capsules, powders, granules, suspensions or solutions. The compositions can also contain suitable preservatives, coloring and flavoring agents, or agents that produce slow release. Potential carriers that can be used in the preparation of the pharmaceutical compositions of this invention include, but are not limited to, gelatin capsules, sugars, cellulose derivations such as sodium carboxymethyl cellulose, gelatin, talc, magnesium stearate, vegetable oil such as peanut oil, etc., glycerin, sorbitol, agar and water. Carriers may also serve as a binder to facilitate tabletting of the compositions for convenient administration.

7. EXAMPLES

The following examples are presented for purposes of illustration only and are not intended to limit the scope of the invention in any way.

7.1. EXAMPLE: PRODUCTION OF LT(R192G)

The wild-type LT toxin is encoded on a naturally occurring plasmid found in strains of enterotoxigenic *E. coli* capable of producing this toxin. Clements et al. had previously cloned the LT gene from a human isolate of *E. coli* designated H10407. This subclone consists of a 5.2 kb DNA fragment from the enterotoxin plasmid of H10407 inserted into the *Pst*I site of plasmid pBR322 (Clements et al, 1983, *Infect. Immun.* 40:653). This recombinant plasmid, designated pDF82, has been extensively characterized and expresses LT under control of the native LT promoter. From pDF82, Clements et al. derived plasmid pBD95, which is fully described in PCT Publication WO96/06627.

Figure 1A shows the construction of plasmid pCS95, which was constructed by inserting the native LT-A subunit regulatory region upstream from the LT-A coding region of

pBD95. Figure 1B shows the Arg to Gly mutation at position 192. The BamHI and XbaI restriction sites referred to in the diagram as "new" were added by site directed mutagenesis, as described in PCT Publication WO96/06627. The new XbaI site was added through a silent mutation, resulting in no alteration of the amino acid sequence of the peptide encoded by the gene.

LT(R192G) was then purified by agarose affinity chromatography from bacteria expressing pCS95. This mutant LT, designated LT(R192G) was then examined by SDS-polyacrylamide gel electrophoresis for modification of the trypsin sensitive bond. Samples were examined with and without exposure to trypsin and compared with native (unmodified) LT. LT(R192G) does not dissociate into A_1 and A_2 when incubated with trypsin, thereby indicating that sensitivity to protease has been removed.

7.2. EXAMPLE: PRODUCTION OF LT-B

The wild-type LT toxin is encoded on a naturally occurring plasmid found in strains of enterotoxigenic *E. coli* capable of producing this toxin. Clements et al. had previously cloned the LT gene from a human isolate of *E. coli* designated H10407. This subclone consists of a 5.2 kb DNA fragment from the enterotoxin plasmid of H10407 inserted into the *Pst*I site of plasmid pBR322 (Clements et al, 1983, *Infect. Immun.* 40:653). This recombinant plasmid, designated pDF82, has been extensively characterized and expresses LT under control of the native LT promoter. The next step in this process was to place the LT-B gene under the control of a strong promoter, in this case the *lac* promoter on plasmid pUC18. This was accomplished by isolating the gene for LT-B from pDF87 and recombining it in a cassette in the vector plasmid. This plasmid, designated pJC217, is fully described in U.S. Patent No. 5,308,835.

LT-B was then purified by agarose affinity chromatography from bacteria expressing plasmid pJC217.

7.3. EXAMPLE: LT(R192G) AND FREE B-SUBUNIT IN THE PATENT MOUSE ENTEROTOXICITY ASSAY

LT(R192G) with no free B-subunit was admixed with different ratios of B-subunit and examined for toxicity in the patent mouse assay. The results are shown in Figure 2. In a second experiment, free B-subunit was admixed with LT(R192G) and also with native LT at a ratio of either 3:1 or 10:1. The results are shown in Figure 3.

7.4. EXAMPLE: EFFECT OF FREE B-SUBUNIT ON INTRANASAL ADJUVANTICITY

The effect of administration of LT(R192G) with excess free B-subunit on both intranasal and oral adjuvanticity was examined by administration of an illustrative antigen with the adjuvant composition to different mucosal surfaces.

Ovalbumin (Ova) was selected as a representative antigen for these studies. A number of investigations, including our own (Clements et al., 1988, *Vaccine* 6:269-277; Dickinson and Clements, 1996, *Mucosal Vaccines* __:73-87; Dickinson and Clements, 1995, *Infection and Immunity* 63:1617-1623; Tsuji et al., 1997, *Immunology* 90:176-182; Yamamoto et al., 1997, *Journal of Experimental Medicine* 185:1203-1210; Yamamoto et al., 1997, Proceedings of the National Academy of *Sciences* 94:5267-5272; DiTommaso et al., 1996, *Infection and Immunity* 64:974-979; Douce et al., 1995, *Proceedings of the National Academy of Sciences* 92:1644-1648; Douce et al., 1997, *Infection and Immunity* 65:2821-2828), have used this protein and it provides a useful reference for comparison to other studies. For those studies, both serum anti-Ova IgG and Ova-specific T-cell responses were examined.

A second antigen, Colonizing Factor Antigen I (CFAI) of enterotoxigenic *E. coli* was included in one set of experiments. In these studies, serum anti-CFAI was examined because anti-CFAI antibodies may be protective against diarrheal disease caused by there organisms.

In the first series of experiments, mice were immunized intranasally with Ova alone or in conjunction with 5 μg of LT(R192G) or 1.25 μg of LT(R192G) plus 3.75 μg of free B-subunit, designated 1AB5:3B5. Serum anti-Ova IgG was determined by ELISA. There were seven animals per group and the means for each data point are shown. As shown in Figure 4, mice immunized intranasally with Ova in conjunction with LT(R192G) containing excess B-subunit had serum anti-Ova IgG responses indistinguishable from animals immunized with Ova in conjunction with LT(R192G) without excess of B-subunit, even though a significantly lower total amount of LT(R192G) was administered (1.25 μg vs. 5 μg). This demonstrates that excess free B-subunit is able to enhance the adjuvanticity of LT(R192G). When the Ova-specific T-cell responses from these animals were examined, both the Th1/IFN-gamma (Figure 5) and Th2/IL-10 (Figure 6) anti-Ova responses were equivalent when free B-subunit was included in the adjuvant formulation compared to LT(R192G) without free B-subunit. There is no significant difference in the IFN-gamma and IL-10 responses between these two groups.

7.5. EXAMPLE: EFFECT OF FREE B-SUBUNIT ON ORAL ADJUVANTICITY

In the next series of experiments, mice were immunized orally with purified Colonizing Factor I (CFAI) from enterotoxigenic *E. coli* in conjunction with 6.25 μg of LT(R192G) or 6.25 μg of LT(R192G) plus 18.75 μg of free B-subunit, designated 1AB5:3B5. Serum anti-CFAI IgG was determined by ELISA. There were seven animals per group and the means for each data point are shown. As shown in Figure

7, mice immunized orally with CFAI in conjunction with LT(R192G) containing excess B-subunit had serum anti-CFAI IgG responses significantly higher that did animals immunized with CFAI in conjunction with LT(R192G) without excess of B-subunit. This demonstrates that excess B-subunit is able to enhance the immune response elicited by oral administration of LT(R192G) with an antigen. One possibility was that the CFAI response was unique because of the inherent ability of colonizing factors to bind to epithelial cells. To further elucidate this unexpected finding, a third series of experiments was performed.

In this series of experiments, mice were immunized orally with Ova alone or in conjunction with 25 μg of LT(R192G) or 6.25 μg of LT(R192G) plus 18.75 μg of free B-subunit, designated 1AB5:3B5. Serum anti-Ova IgG was determined by ELISA. There were ten animals per group and the means for each data point are shown. As shown in Figure 8, mice immunized orally with Ova in conjunction with LT(R192G) containing excess B-subunit had significantly higher serum anti-Ova IgG responses than did animals immunized with Ova in conjunction with LT(R192G) without excess of B-subunit, even though a significantly lower total amount of LT(R192G) was administered (6.25 μg vs. 25 μg). This finding was consistent with the enhanced anti-CFAI serum IgG responses observed when excess B-subunit was included in the oral adjuvant formulation with CFAI as the antigen (Figure 7). When the Ova-specific T-cell responses from these animals were examined, both the Th1/IFN-gamma (Figure 9) and Th2/IL-10 (Figure 10) anti-Ova responses were qualitatively different and quantitatively enhanced when free B-subunit was included in the adjuvant formulation compared to LT(R192G) without free B-subunit.

These findings demonstrate that when LT(R192G) is used as an oral adjuvant, the presence of free B-subunit

elicits an antigen-specific T-cell response, a response which is substantially non-existent when LT(R192G) is used without excess free B-subunit. This represents a qualitative change in the type of immune response elicited. These findings also illustrate that excess free B-subunit enhances the humoral immune response elicited by LT(R192G) when used as an oral adjuvant; this represents a quantitive change in response.

8. DEPOSIT OF MICROORGANISMS

The following microorganism containing the designated plasmid was deposited with the American Type Culture Collection (ATCC), (present address: 1081 University Boulevard, Manassas, VA 20110-2209)on March 13, 1998, and has been assigned the indicated accession number:

Microorganism	Accession Number
E. coli JM83 (pCS 95)	98696

Although the invention is described in detail with reference to specific embodiments thereof, it will be understood that variations which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 31, lines 15-16 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet

Name of depositary institution

American Type Culture Collection

Address of depositary institution (including postal code and country)

10801 University Blvd.
Manassas, VA 20110-2209
US

Date of deposit March 13, 1998 Accession Number 98696

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the International application when filed (to be checked by the receiving Office)

_____
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau

was

_____
(Authorized Officer)

Form PCT/RO/134 (January 1981)

WHAT IS CLAIMED IS:

1. A composition comprising an admixture of a mutant *E. coli* heat-labile enterotoxin holotoxin, in which arginine at amino acid position 192 is replaced with glycine, and *E. coli* heat-labile enterotoxin B-subunit, said B-subunit being free of A-subunit and in an amount sufficient to result in a ratio of 1:1 to 100:1 of B-subunit to mutant holotoxin.

2. The composition according to claim 1, in which the ratio of B-subunit to mutant holotoxin is 2:1 to 10:1.

3. The composition according to claim 1, in which the ratio of B-subunit to mutant holotoxin is about 3:1.

4. A vaccine preparation comprising an antigen in combination with the composition according to claim 1, 2 or 3.

5. The vaccine preparation according to claim 4, in which the antigen is selected from the group consisting of bacterial, viral, protozoal, fungal, helminthal, and other microbial antigens.

6. The vaccine preparation according to claim 4, in which the antigen is selected from the group consisting of antigens of: *Streptococcus pyogenes, Streptococcus pneumoniae, Neisseria gonorrhoea, Neisseria meningitidis, Corynebacterium diphtheriae, Clostridium botulinum, Clostridium perfringens, Clostridium tetani, Haemophilus influenzae, Klebsiella pneumoniae, Klebsiella ozaenae, Klebsiella rhinoscleromotis, Staphylococcus aureus, Vibrio cholerae, Escherichia coli, Pseudomonas aeruginosa, Campylobacter (Vibrio) fetus, Campylobacter jejuni, Aeromonas*

hydrophila, Bacillus cereus, Edwardsiella tarda, Yersinia enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Salmonella typhimurium, Treponema pallidum, Treponema pertenue, Treponema carateneum, Borrelia vincentii, Leptospira icterohemorrhagiae, Mycobacterium tuberculosis, Toxoplasma gondii, Pneumocystis carinii, Francisella tularensis, Brucella abortus, Brucella suis, Brucella melitensis, Mycoplasma spp., Rickettsia prowazeki, Rickettsia tsutsugumushi, Chlamydia spp., Helicobacter pylori, Coccidioides immitis, Aspergillus fumigatus, Candida albicans, Blastomyces dermatitidis, Cryptococcus neoformans, Histoplasma capsulatum, Entomoeba histolytica, Trichomonas tenas, Trichomonas hominis, Trichomonas vaginalis, Trypanosoma gambiense, Trypanosoma rhodesiense, Trypanosoma cruzi, Leishmania donovani, Leishmania tropica, Leishmania braziliensis, Pneumocystis pneumonia, Enterobius vermicularis, Trichuris trichiura, Ascaris lumbricoides, Trichinella spiralis, Strongyloides stercoralis, Schistosoma japonicum, Schistosoma mansoni, Schistosoma haematobium, variola virus, vaccinia virus, cowpox virus, varicella-zoster virus, Herpes Simplex virus 1, Herpes Simplex virus 2, influenza viruses, parainfluenza virus, mumps, measles, respiratory syncytial virus, rubella, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis E virus, and Non-A/Non-B Hepatitis virus antigens.

7. A composition useful in producing a protective immune response to a pathogen in a host comprising an admixture of an effective amount of an antigen and an adjuvant effective amount of the composition according to claim 1, 2 or 3.

8. A kit useful in producing a protective immune response in a host to a pathogen comprising two components: (a) an effective amount of antigen and (b) an admixture of an adjuvant effective amount of a mutant *E. coli* heat-labile enterotoxin holotoxin, in which arginine at amino acid position 192 is replaced with glycine, and an amount of *E. coli* heat-labile enterotoxin B-subunit, said B-subunit being free of holotoxin and in an amount sufficient to result in a ratio of 1:1 to 100:1 of B-subunit of mutant holotoxin.

9. A kit useful in producing a protective immune response in a host to a pathogen comprising two components: (a) an effective amount of antigen and (b) an admixture of an adjuvant effective amount of a mutant *E. coli* heat-labile enterotoxin holotoxin, in which arginine at amino acid position 192 is replaced with glycine, and an amount of *E. coli* heat-labile enterotoxin B-subunit, said B-subunit being free of holotoxin and in an amount sufficient to enhance the adjuvanticity of said mutant *E. coli* heat-labile enterotoxin holotoxin, wherein said components are in a pharmaceutically acceptable carrier and said components may be administered either after having been mixed together or separately within a short time of each other.

10. A method of creating or sustaining a protective or adaptive immune response to an antigen in a host comprising administering an admixture of an effective amount of an antigen, an adjuvant effective amount of a mutant *E. coli* heat-labile enterotoxin holotoxin in which arginine at amino acid position 192 is replaced with glycine, and an amount of a *E. coli* heat-labile enterotoxin B-subunit, said B-subunit being free of holotoxin and in an amount sufficient to enhance the adjuvanticity of said mutant *E.*

coli heat-labile enterotoxin holotoxin, in an acceptable pharmaceutical carrier.

11. The method of claim 10 where the administration is oral.

12. The method of claim 10 where the administration is nasal.

13. The method of claim 10 where a serum response is produced.

14. The method of claim 10 where a mucosal response is produced.

15. The method of claim 10 further comprising administering a subsequent boost of the antigen.

16. The method of claim 10 wherein the antigen is derived from the group consisting of bacteria, viruses, protozoa, fungi, helminths, and other microbial pathogens.

17. A method of inducing a protective immune response to an antigen in a host comprising orally administering an effective amount of an antigen, an adjuvant effective amount of a mutant *E. coli* heat-labile enterotoxin holotoxin, in which arginine at amino acid position 192 is replaced with glycine, and an amount of a *E. coli* heat-labile enterotoxin B-subunit, said B-subunit being free of holotoxin and in an amount sufficient to result in a ratio of 1:1 to 100:1 B-subunit to holotoxin, in an orally acceptable pharmaceutical carrier.

18. The method of claim 17 in which the antigen, the mutant holotoxin, and the B-subunit are administered simultaneously.

19. The method of claim 17 in which the antigen, the mutant holotoxin, and the B-subunit are administered separately within a short time of each other.

20. The method of claim 17 in which the antigen is administered separately within a short time of the simultaneous administration of the mutant holotoxin and the holotoxin free B-subunit.

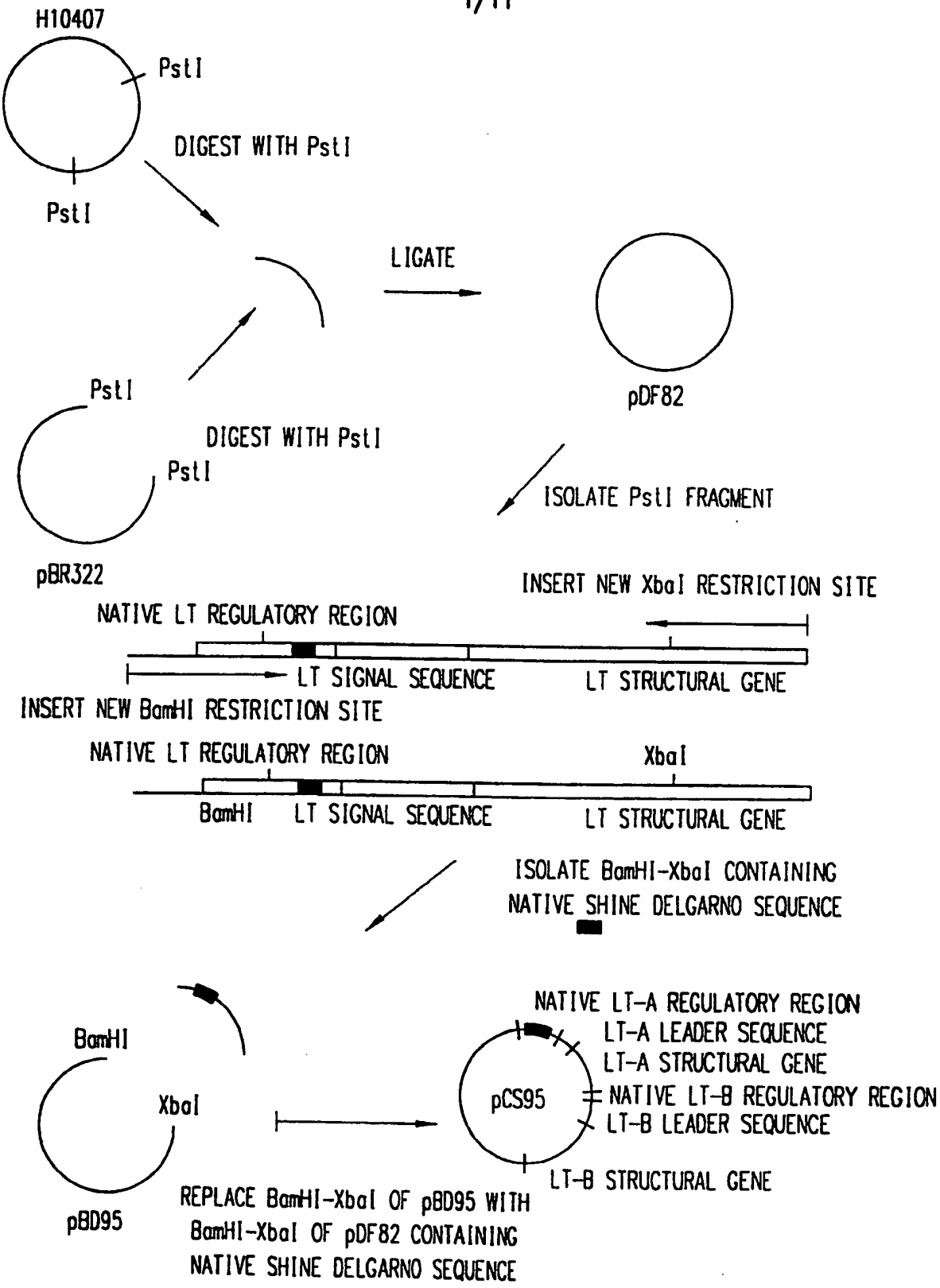
H10407
PstI
DIGEST WITH PstI
PstI
LIGATE
pDF82
PstI
DIGEST WITH PstI
PstI
ISOLATE PstI FRAGMENT
pBR322
INSERT NEW XbaI RESTRICTION SITE
NATIVE LT REGULATORY REGION
LT SIGNAL SEQUENCE
LT STRUCTURAL GENE
INSERT NEW BamHI RESTRICTION SITE
NATIVE LT REGULATORY REGION
XbaI
BamHI
LT SIGNAL SEQUENCE
LT STRUCTURAL GENE
ISOLATE BamHI-XbaI CONTAINING
NATIVE SHINE DELGARNO SEQUENCE
NATIVE LT-A REGULATORY REGION
LT-A LEADER SEQUENCE
LT-A STRUCTURAL GENE
NATIVE LT-B REGULATORY REGION
LT-B LEADER SEQUENCE
LT-B STRUCTURAL GENE
pCS95
BamHI
XbaI
pBD95
REPLACE BamHI-XbaI OF pBD95 WITH
BamHI-XbaI OF pDF82 CONTAINING
NATIVE SHINE DELGARNO SEQUENCE

FIG. 1A

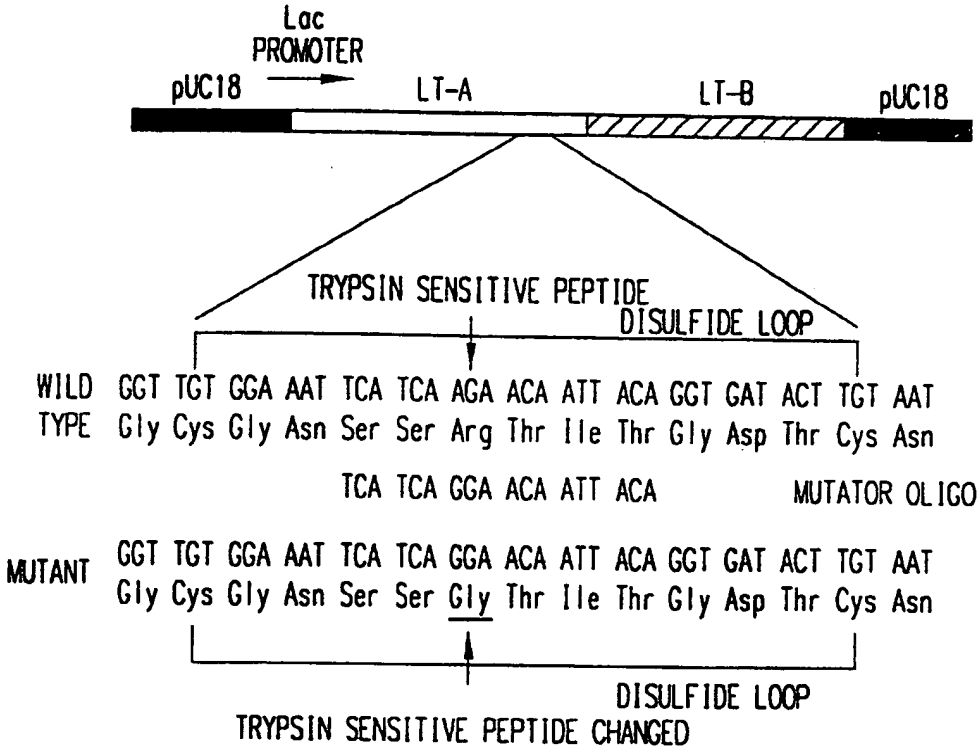
Lac
PROMOTER
pUC18
LT-A
LT-B
pUC18
TRYPSIN SENSITIVE PEPTIDE
DISULFIDE LOOP
WILD TYPE GGT TGT GGA AAT TCA TCA AGA ACA ATT ACA GGT GAT ACT TGT AAT
Gly Cys Gly Asn Ser Ser Arg Thr Ile Thr Gly Asp Thr Cys Asn
TCA TCA GGA ACA ATT ACA
MUTATOR OLIGO
MUTANT GGT TGT GGA AAT TCA TCA GGA ACA ATT ACA GGT GAT ACT TGT AAT
Gly Cys Gly Asn Ser Ser Gly Thr Ile Thr Gly Asp Thr Cys Asn
DISULFIDE LOOP
TRYPSIN SENSITIVE PEPTIDE CHANGED

FIG. 1B

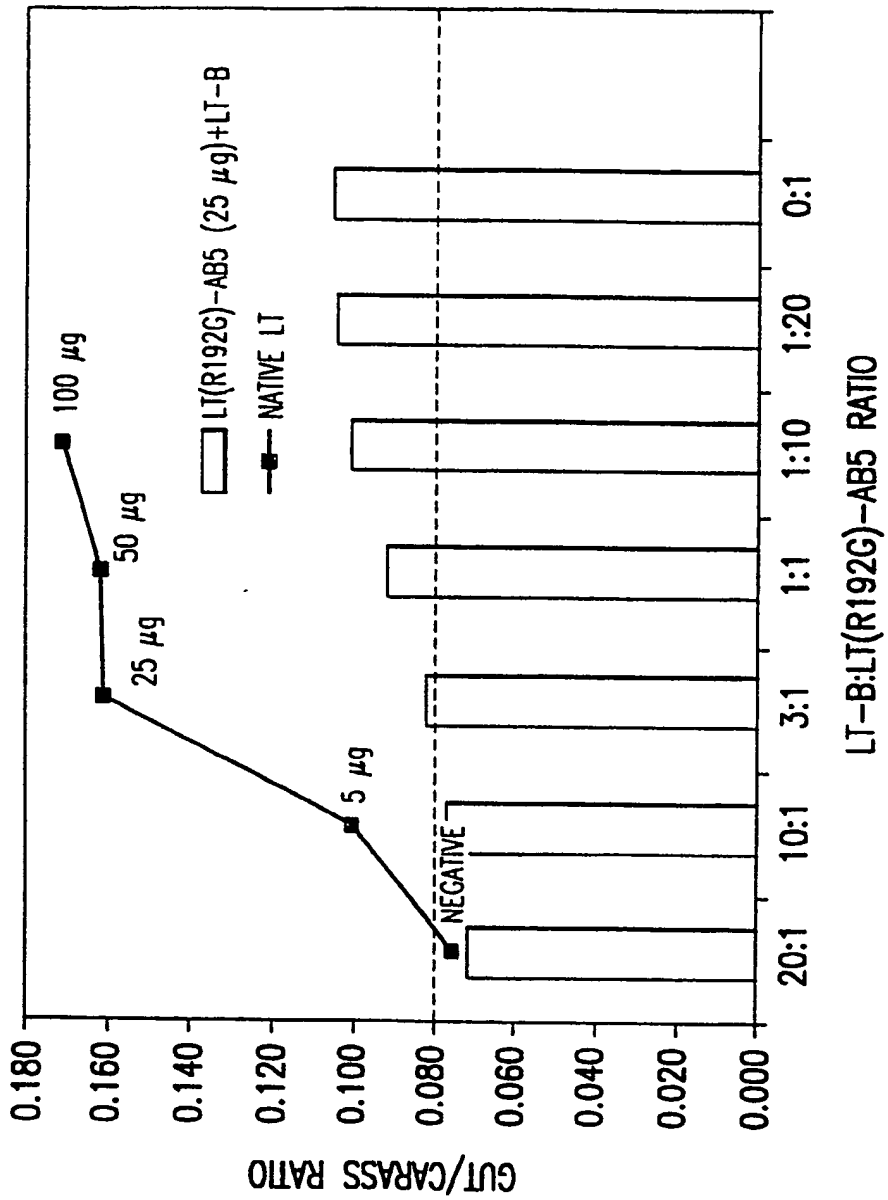
GUT/CARASS RATIO
0.180
0.160
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0.000
LT(R192G)-AB5 (25 μg)+LT-B
NATIVE LT
100 μg
50 μg
25 μg
5 μg
NEGATIVE
20:1
10:1
3:1
1:1
1:10
1:20
0:1
LT-B:LT(R192G)-AB5 RATIO

FIG.2

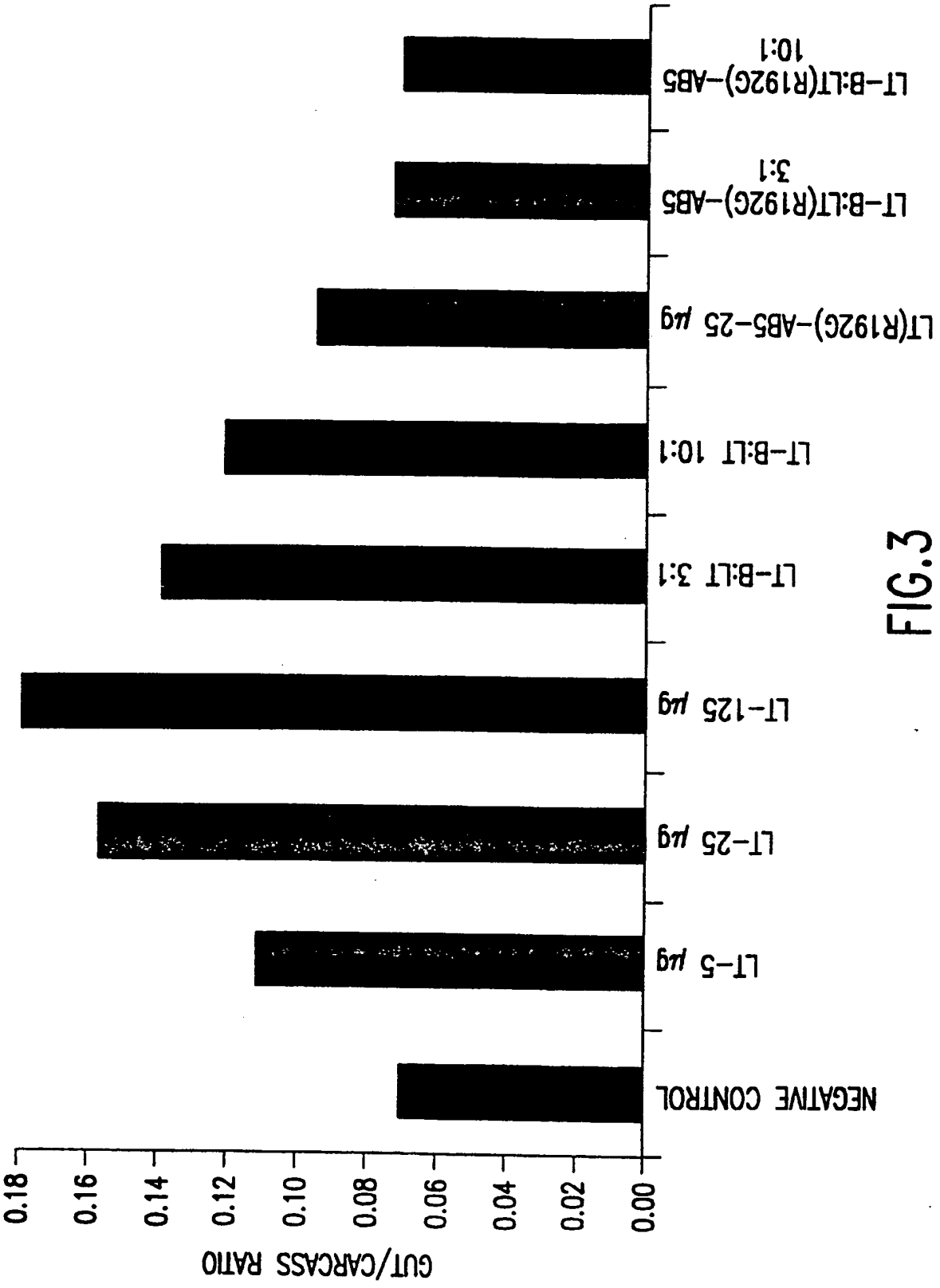
GUT/CARCASS RATIO
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
NEGATIVE CONTROL
LT-5 μg
LT-25 μg
LT-125 μg
LT-B:LT 3:1
LT-B:LT 10:1
LT(R192G)-AB5-25 μg
LT-B:LT(R192G)-AB5 3:1
LT-B:LT(R192G)-AB5 10:1

FIG.3

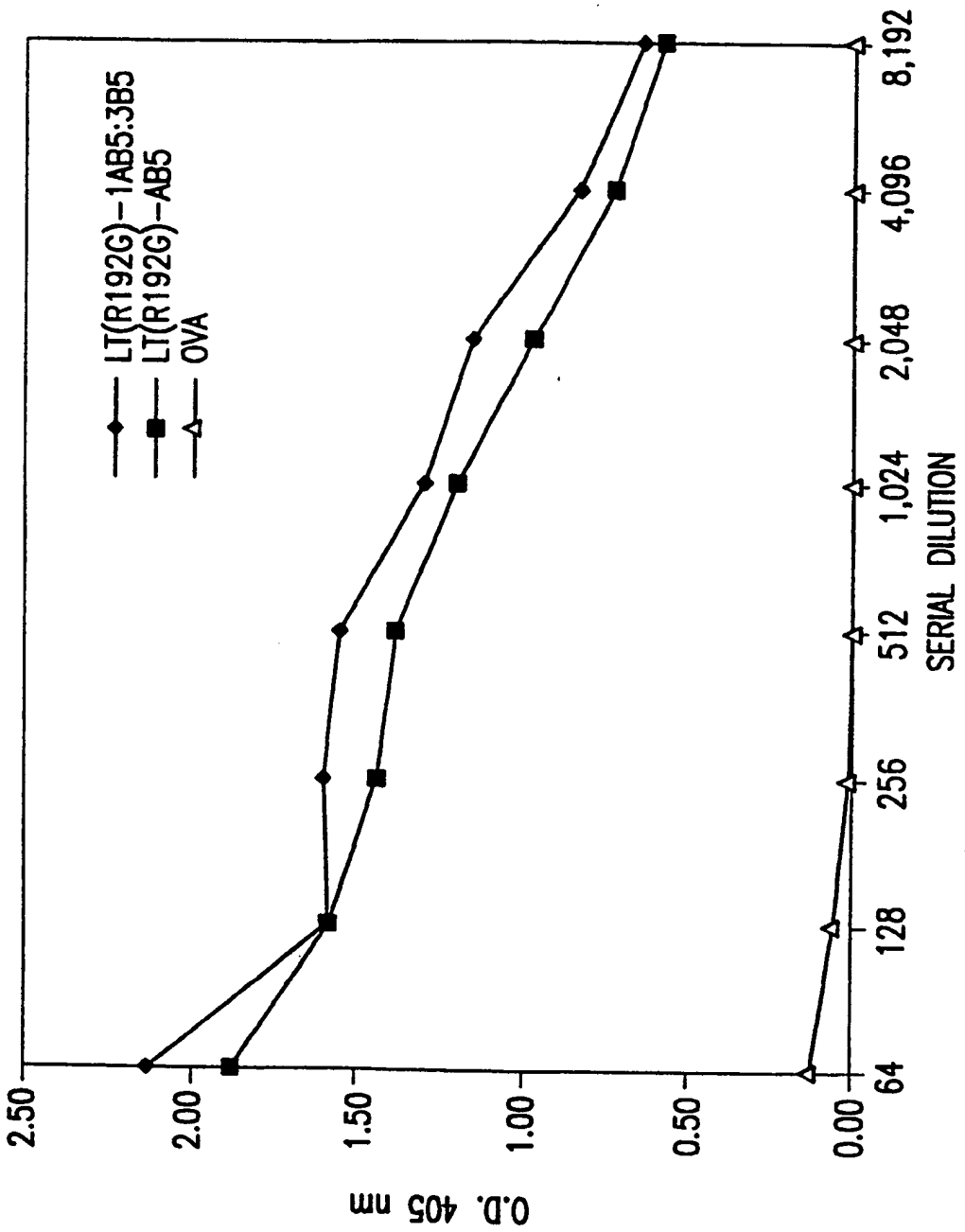
LT(R192G)-1AB5:3B5
LT(R192G)-AB5
OVA
O.D. 405 nm
2.50
2.00
1.50
1.00
0.50
0.00
64
128
256
512
1,024
2,048
4,096
8,192
SERIAL DILUTION

FIG.4

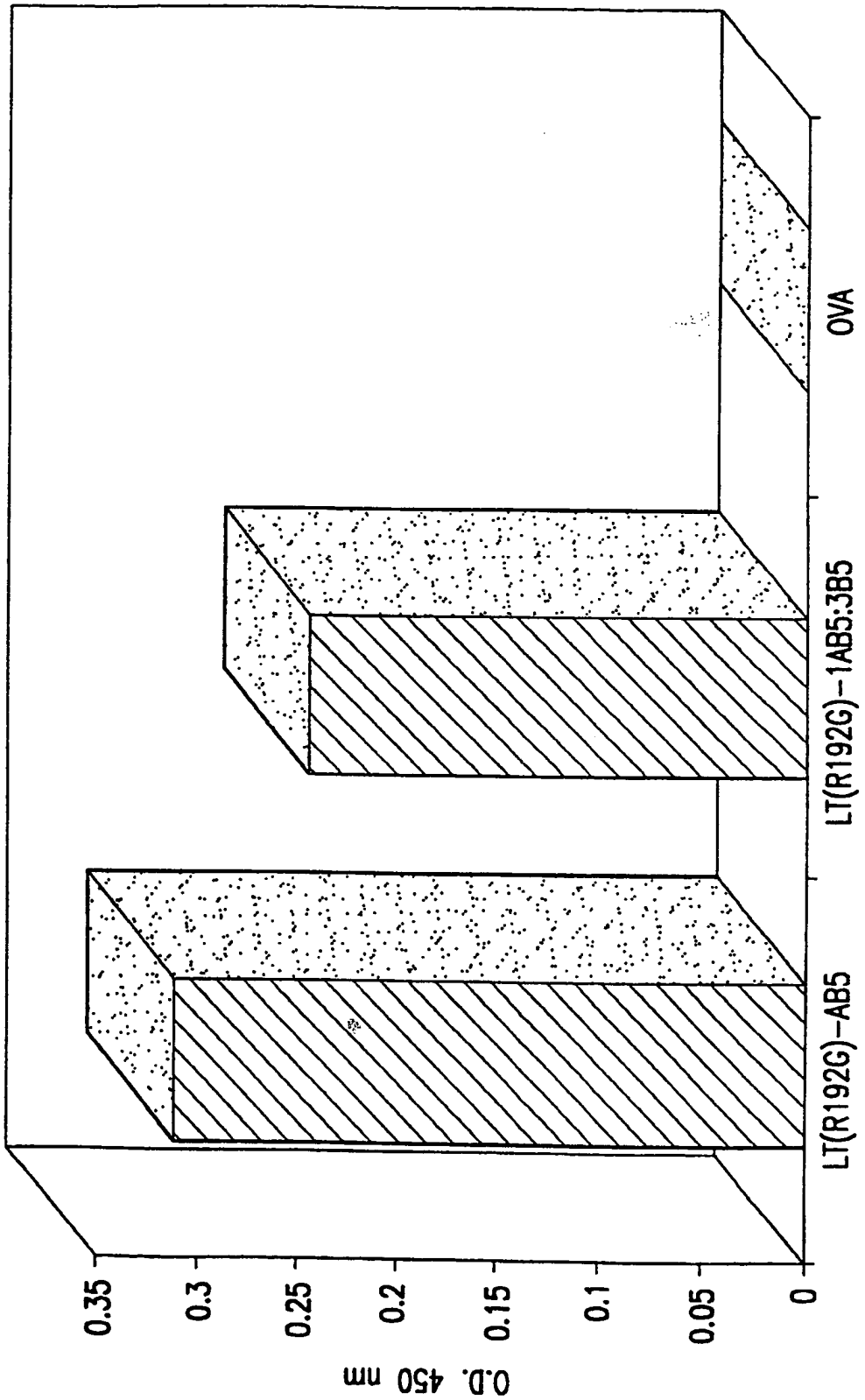
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
O.D. 450 nm
LT(R192G)-AB5
LT(R192G)-1AB5:3B5
OVA

FIG.5

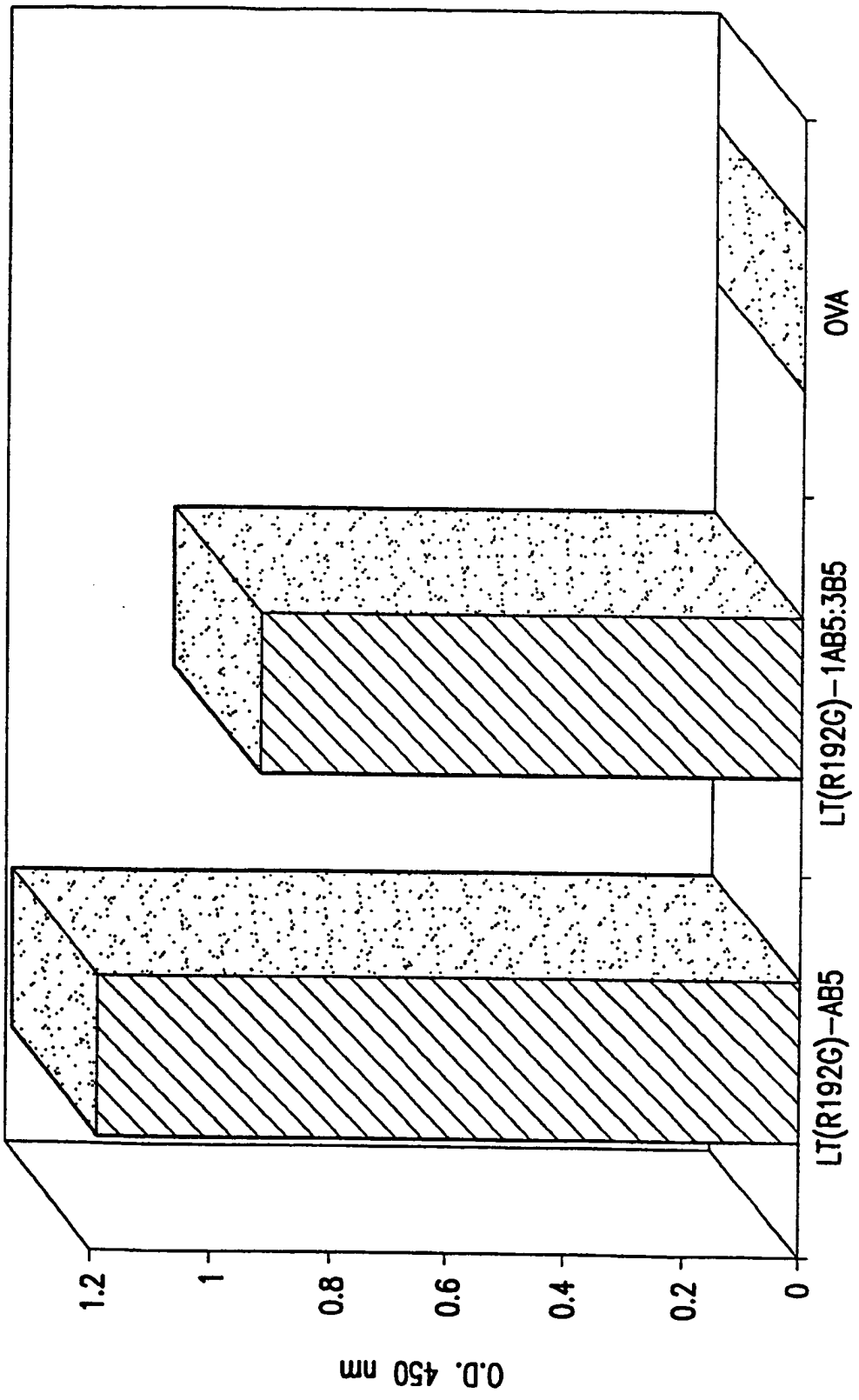

FIG.6

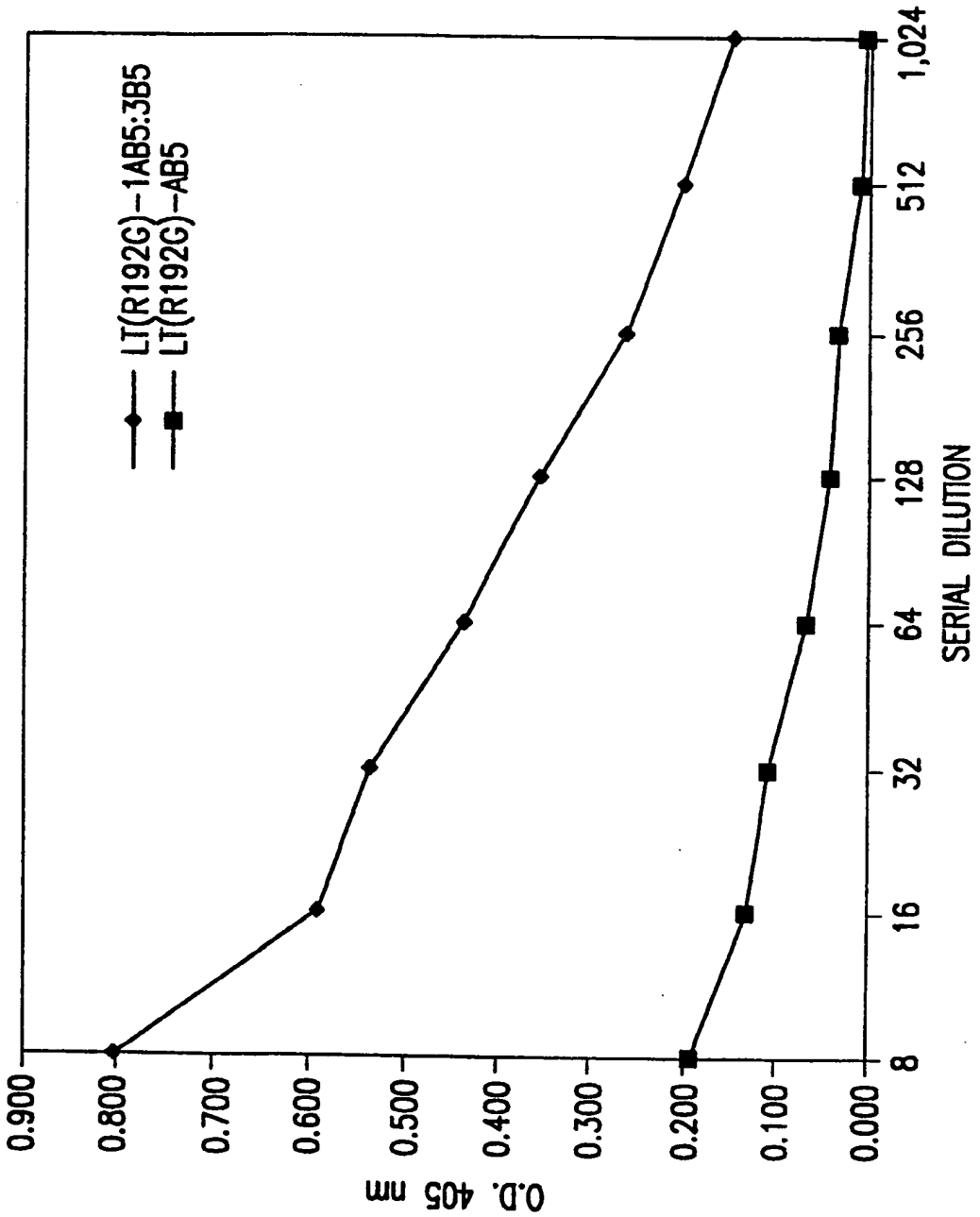
LT(R192G)-1AB5:3B5
LT(R192G)-AB5
O.D. 405 nm
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
8
16
32
64
128
256
512
1,024
SERIAL DILUTION

FIG.7

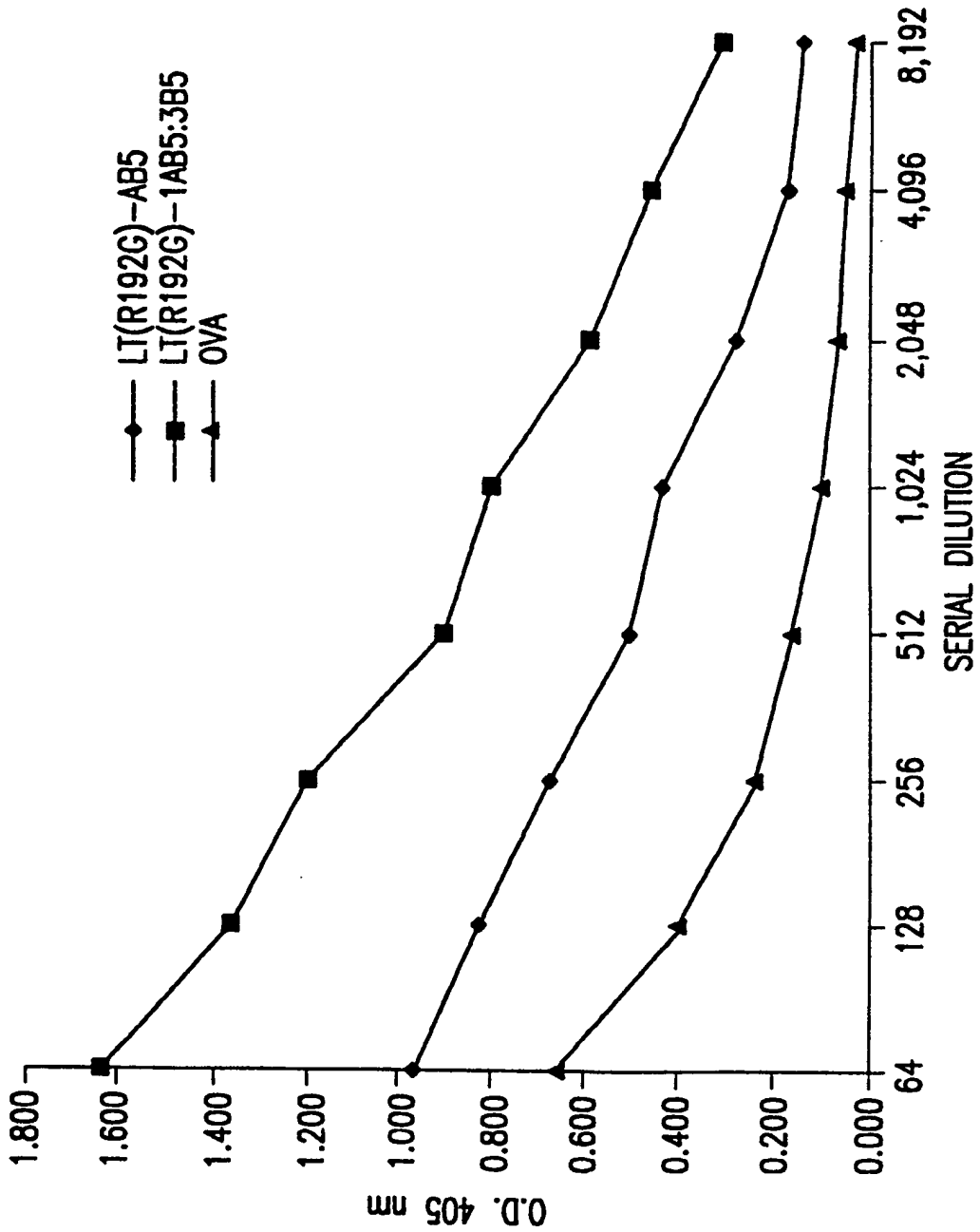
LT(R192G)-AB5
LT(R192G)-1AB5:3B5
OVA
O.D. 405 nm
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
64
128
256
512
1,024
2,048
4,096
8,192
SERIAL DILUTION

FIG.8

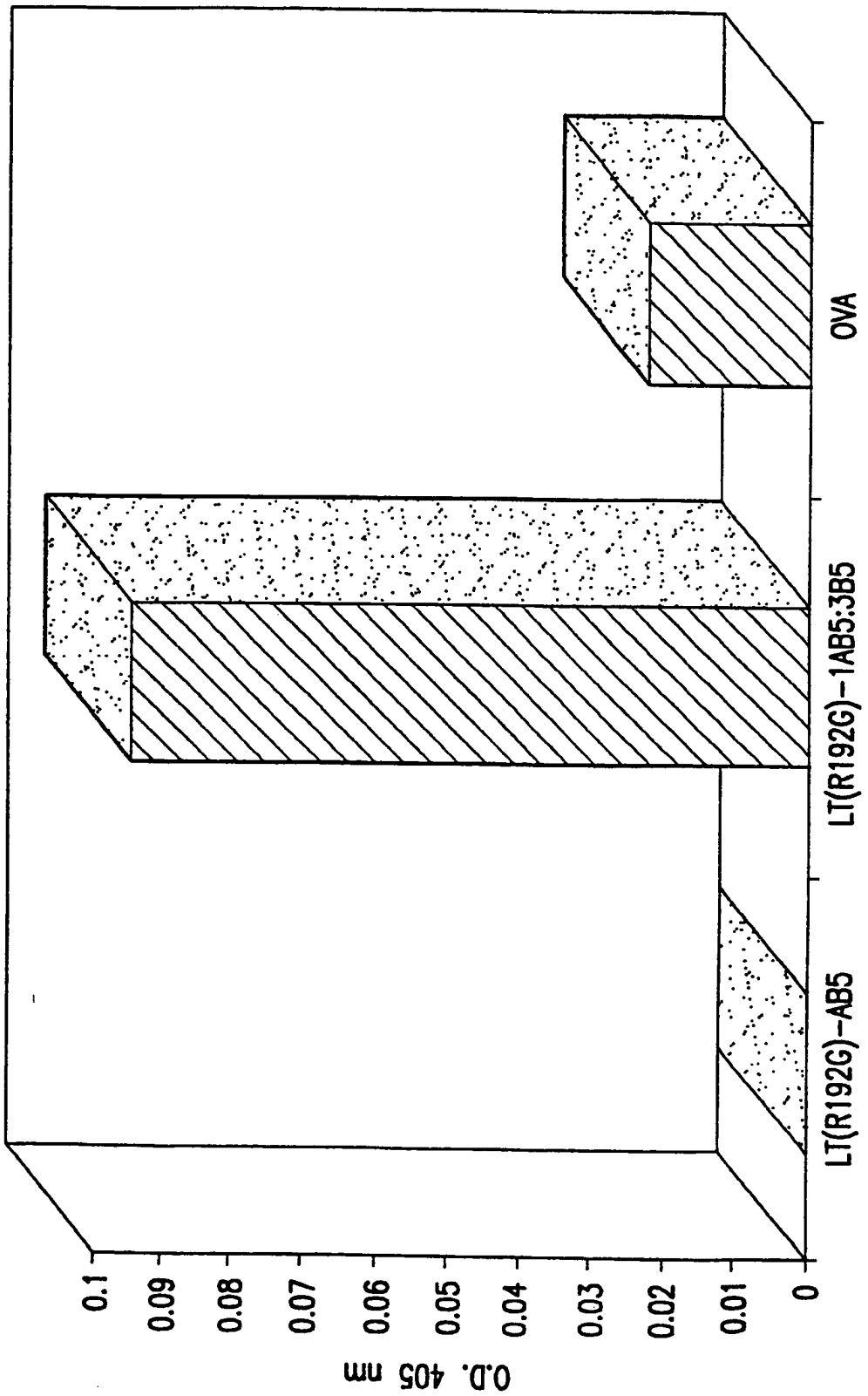
O.D. 405 nm
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.1
LT(R192G)-AB5
LT(R192G)-1AB5:3B5
OVA

FIG.9

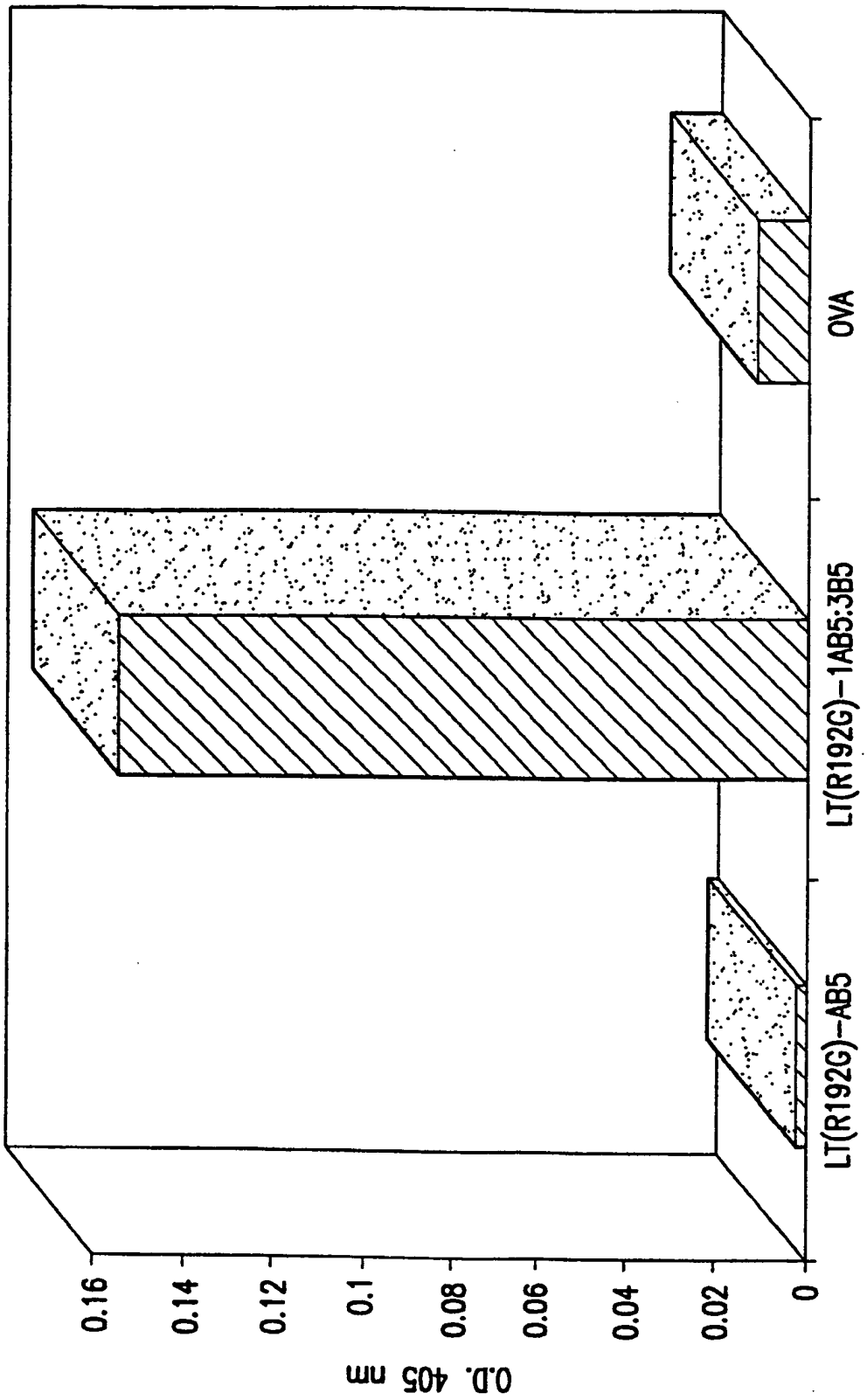
O.D. 405 nm
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
LT(R192G)-AB5
LT(R192G)-1AB5:3B5
OVA

FIG.10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05622

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 39/00, 39/116, 39/108; G01N 33/53
US CL :424/184.1, 203.1, 241.1; 435/975
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 203.1, 241.1; 435/975

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MEDLINE, BIOSIS, EMBASE, ALLSCIENCE
search terms: e(1w)coli, escherichia (1w) coli, b(1w)subunit, lt, R192G, adjuvant?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GIANNELLI et al. Protease Susceptibility and Toxicity of Heat-labile Enterotoxins with a Mutation in the Active Site or in the Protease-sensitive Loop. Infection and Immunity. January 1997, Vol. 65, No. 1, pages 331-334, see entire document.	1-20
A	US 4,808,700 A (ANDERSON et al) 28 February 1989, see entire document.	1-20
A	US 5,308,835 A (CLEMENTS et al) 03 May 1994, see entire document.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
23 APRIL 1999

Date of mailing of the international search report
13 MAY 1999

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